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SENSOR FOR DETECTING PROTEINS AND PROCESS FOR ITS PRODUCTION

[Sensor zum Nachweis von Proteinen und

Verfahren zu dessen Herstellung]

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This invention relates to a sensor for the detection of proteins according to the preamble of Claim 1, such as it is known from DI 44 18 926 and a process for its production.

Until now, there have been some established analytical methods for analyzing also biomolecules, which, however, employ mostly laborious and expensive laboratory methods (HPLC, GC-MS). A similar reasonably priced alternative to the method described here, on the other hand, is represented by the so-called immunoassays. They are also based on the binding of an analyt upon an antibody, but they necessarily employ an indirect procedure for the purpose of detecting this bond. Here, one adds to the sample a radioactively marked, fluorescence-marked or enzyme-marked analyt-analogous molecule that competes with the analyt for the antibody bonding points. For analysis purposes, this means that one needs a process that consists of several reagent supplies, incubation steps and washing steps; the total time per assay typically is one hour. This means that on-line measurement is impossible.

Moreover, other immunosensor principles are described in the literature on the subject. In the opinion of many authors, they still deviate greatly from the ideal concept of a reasonably priced, sufficiently sensitive future immunosensor: Several

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[1]Numbers near the margin indicate pagination in the foreign text.

general articles have criticized such methods already on the basis of their high cost (surface plasmon resonance, grid couplers, differential interferometer) or due to their low sensitivity (potentiometric immunosensor), while the immunosensor on a surface wave basis has been favored so far.

EP 0 361 729 A2 discloses a method of the above-mentioned kind for the production of a sensor that has a protective layer for spatial separation of substrate and aqueous analyt solution. /2

At a working frequency of > 100 MHz, this sensor displays attenuations of between 30 and 40 dB, which causes it to be greatly prone to trouble, with intensive background noise.

The disadvantage of the sensor described in DE 44 18 926 resides in the fact that the production demands much labor and entails a high danger potential and that the sensor properties are widely scattered.

The object of the invention is so to design a sensor of the above-mentioned kind that it can be produced in a simple manner and that it will be well reproducible.

This problem is solved by the features given in Claims 1 and 3. The subclaims describe advantageous developments of the process.

The sensor facilitates the specific measurement of the presence or concentration of various biomolecules such as

proteins and enzymes as well as more complex macromolecules -- parts of the genotype (DNS, RNS) or various pathological agents (for example, viruses or bacteria) -- by means of direct evidence as to the bonding to specific antibodies in aqueous solutions. With this method, we therefore no longer need any time-consuming procedures that are based on competition between labeled and unlabeled analyts (indirect detection process in conjunction with immunoassays).

The sensor is a mass-sensitive sensor that uses the sound speed change of acoustic surface waves caused during the sorption of the analyt in order to draw conclusions as to the sorbed mass of the analyt and thus the latter's presence of concentration on the solution.

To get a specifically analyt-related sorption reaction, we need selective coatings on the surface wave substrate. Special flexibility is guaranteed here when these coatings consist of

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immunochemically active molecules such as antibodies or antigens. The invention-based sensor thus is a genuine immunosensor that determines its data in-situ and thus facilitates a genuine on-line measurement method for bioanalytics.

The described sensor offers a series of advantages compared to conventional bioanalytics:

- reasonably priced: 4-10 DM [German marks] each;

- sensitivity same as bioassay;
- can be transferred to any biosystems;
- very good long-term stability.

The invention will be described in greater detail below with the help of an example and referring to the figures.

Figure 1 shows the course of enzymatic glucose decomposition on a sensor and

Figure 2 shows the course of immunoreactions for differing antibody concentrations.

Figure 3 shows the TRIMID content of the receptor protein and

Figure 4 shows the latter's enzyme activity on the basis of absorption spectra.

Figure 5 shows the receptor bonding to the polymer polyimide.

The sensor in our example works on the basis of acoustic surface waves. Such a sensor is described in DE 43 19 215.

The sensor body is coated on one side, first of all, with a polymer, in this example an aromatic polyimide. The coating on the surface of the sensor body with polyimide was done as described in DE 44 18 926, page 3, lines 11 to 55. Then 10 µl of the suitably diluted, modified receptor molecules are placed on the polyimidized sensor surface.

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As one can see in Figure 5, there now takes place the immobilization of photomarked enzyme on the polyimidized sensor surface when the water content in the protein matrix is kept low. If the water content in the protein is too high, then upon exposure, there is a reaction of the carbene with the water so that the insertion reaction between the polyimide and the protein is forced into the background and so that there is no bonding on the surface of the sensor. This is why the sensors are treated for 20-40 minutes in a vacuum drying chamber at room temperature and at a pressure of < 10 mbar. The optimum is a 30-minute drying phase at 1 mbar. The resultant dried enzyme film was then illuminated with a mercury vapor lamp. The production of the triplet carbenes requires an illumination of the enzyme film at 348 nm and 0.7 mW/cm^2 for 30 minutes.

Glucose oxidase (T-glucose oxidase, T-GOD), modified with TRIMID with a protein content of 1.92 mg/ml, was used for immobilization. The TRIMID content was 6.5 moles of TRIMID per mole of glucose oxidase.

Preliminary investigations showed that the T-GOD solution could not be separated undiluted on the sensor. When the protein concentration is too high, there are excessively high input attenuations and one cannot observe any acoustic wave.

As a result of these preliminary studies, the T-glucose oxidase solution was diluted in a ratio of 1:125 with phosphate buffer (1:100); next, 10 µl of this solution was placed on the

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sensor and the enzyme was immobilized as described above (vacuum-treated, illuminated).

The quantity of enzyme separated on the sensor surface was determined spectroscopically by means of an enzymatic assay.

The following solutions are necessary to perform the enzyme activity test:

Solution 1

53 mg of 3,5-dichlor-2-hydroxy-benzosulfonic acid are dissolved in water (bidest.) and are brought to a pH of 7 with 1 M NaOH. Then one adds 3 mg of peroxidase (horseradish) and it is filled up with water to a volume of 10 ml.

Solution 2

16.2 mg of 4-aminophenazone are dissolved in 10 ml of water.

Solution 3

1.4 g of Na_2HPO_4 * $2H_2O$ and 700 mg of NaH_2PO_4 as well as 37.2 mg of EDTA are dissolved in 100 ml of water.

Solution 4

1.8 g of -D-glucose is dissolved in 10 ml of water.

Then one puts the following into a bulb:

1.55 ml of Solution 3

0.2 ml of Solution 4

0.2 ml of Solution 1

50 µm of Solution 2

and after thorough mixing (Vortex), one measures the background at 520 nm. Then one adds 50 µl of an appropriately diluted GOD solution and one measures the absorption change at 520 nm (development of a red dye) within the first minutes. In case of high glucose oxidase concentrations, 2 minutes suffice and the

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measurement was continued for 10-20 minutes for the quantities of antigen that were separated on the sensor.

The extinction coefficients of the generated dye amounts to 13300 $(M\ cm)^{-1}$. This means that it is possible to determine the specific enzyme activity of the glucose oxidase solution. This figure is given in terms of "units/mg" and one "unit" is defined as follows: one "unit" oxidizes 1 µmol of -D-glucose per minute at pH = 5.1 (T = 35°C).

The production of 1 mole of the red dye requires 2 moles of hydrogen peroxide (from glucose separation); therefore, the measured increase in the chromophore amounts to 0.5 unit per minute.

The increase in absorption at 520 nm is given by way of example for three different sensors in Figure 1. This shows us a mean value of the increases amounting to 0.0021 absorption unit

per minute. A comparison to the enzyme activity of the T-GOD parent solution shows that this absorption increase corresponds to a protein mass of 18.5 ng [sic] on the entire sensor.

The sensor sensitivity as well as the detection limit for polyclonal antibodies against glucose oxidase was determined by varying the antibody quantity in the analyt flow.

For this purpose, the sensors were coated with T-glucose oxidase via the described photoinitiated reaction and was first flushed with bovine serum albumin (4 mg/ml) in order to block the nonspecific bonding points. The sensors were determined in the throughflow with the protein; therefore, they displayed differing separation speeds, all of which then, however, came to a frequency change of 35 kHz (within an error range of 10%).

The sensors, thus pretreated, were then treated individually with antibody solution samples where the analyt -- polyclonal

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antibody against glucose oxidase -- was conducted in a cycle over the sensors. Different antibody quantities were dissolved here in each case in 5 ml of phosphate buffer and were conducted over the sensors. The concentration series, which is illustrated in the extracts in Figure 2, contained a range of 2-200 µg/ml antibody (corresponding to 10-1,000 µg).

One can recognize various frequency decreases as well as differing initial speeds of the immunoreactions.

The change in the resonance frequency of the oscillator was plotted against the antibody quantity in the analyt flow. The slope of the straight line indicates the sensor sensitivity that was determined as being 58.8 Hz/µg.

The initial speed of the immunoreaction was determined for each measurement in order to determine the correlation between the antibody quantity and the reaction speed. The frequency decrease per unit of time was calculated from the measurement points within the first minute after the addition of the analyt by means of linear regression. The correlation coefficients for all analyzed curves turned out to be definitely greater than 0.98.

The initial speeds thus determined correlate with the quantity of antibody added. This results in a definitely linear connection between the two magnitudes ($r = 0.9822$). The slope of the regression straight line amounts to 4.92 Hz/(sµg).

To determine the detection boundary for polyclonal antibodies against glucose oxidase, one proceeds as follows.

The sensitivity amounts to 58/8 Hz/µg, the axis segment was determined as being 27.1 kHz. Using these values and with reference to the triple noise signal of the sensors (120 Hz), one can determine the detection boundary for polyclonal antibodies against glucose oxidase at 2 µg or 13.6 pmol. The particular antibody quantity was weighed into 5 ml of phosphate buffer;

therefore, this value corresponds to a minimum detectable concentration of 2.7 nmol/l.

The described coating method can also be transferred to other sensor or measurement principles. For example, the sensor chips of the optical grid coupler can be coated equally with modified receptor molecules.

Enzymes, antigens and antibodies as well as nucleic acids can be used as possible receptor molecules.

An example of coating is shown by way of the antigen glucose oxidase that was mixed directly with 3-trifluoromethyl-3-(m-isothiocyanomethyl)-diazirine (TRIMID) in order to get a photoreactive protein. It is basically just as possible to modify the antibody itself with TRIMID, but it is more cost-intensive.

The synthesis route could not be transferred directly from the T-BSA known from the literature to T-GOD because glucose oxidase contains the coenzyme FAD that is not bound to the enzyme in a covalent manner. By mixing GOD with TRIMID at a pH of 11.4 by means of incubation at 50°C, glucose oxidase was, of course, modified with TRIMID, but the coenzyme was separated, something that was evidenced by the ultraviolet spectrum. It was possible to ascertain a shoulder at 348 nm (TRIMID), but the FAD peaks at 375 or 450 nm could no longer be recognized.

Investigations showed that incubation at 50°C leads to the separation of the coenzyme, but that the treatment of the enzyme at pH = 11.4 does not bring about this dissociation.

T-GOD was therefore made according to the following prescription:

18 mg of glucose oxidase and 1.27 g of -D-glucose were dissolved /9

in 0.1% by volume TEA (in water, pH = 11.4) and the resultant solution was set at a pH of 10.4 with pure TEA.

Addition of 170 µl TRIMID (29 µmol/l) in chloroform exposed to ultrasonic waves in the ultrasound bath for 30 seconds, resulting in a milky yellow suspension.

Incubate for 2 hours at 37°C in the water bath.

Chromatograph over a Sephadex G-25 column in 1.5 mM NaCl, 0.05 mM of sodium phosphate buffer (pH = 7.4).

The protein concentration can be determined with the method according to Lowry. For this purpose, we measure a specially prepared BSA standard as reference and we relate to the extinctions of T-GOD to that.

For this investigation, we needed a parent solution consisting of 0.5 ml of 2% $CuSO_4$ solution, 0.5 ml 2% tartrate solution and 49 ml of 2% Na_2CO_3 solution in 0.1 M NaOH. To be able to determine the protein concentration of fractions 12 and 13, we diluted 100 µl with PBS (1:100) to 1 ml and we made 6

samples in that, in each case, 10 µl, 20 µl and 30 µl were diluted down to 200 µl with PBS (1:100) (double determination). To these samples, we added 1 ml each of the parent solution and the mixture was allowed to stand for 10 minutes.

We then added 100 µl 0.5 N Folin solution that generates an extinction maximum at 578 nm and which could be measured after 30 minutes. With the help of the BSA standard, we were then able to calculate the protein quantity of T-GOD; we arrived at a protein mass concentration of 4.22 mg/ml.

The Trimid content of a protein can be checked out by the extinction difference of a sample before and after illumination at 348 nm. In the case of glucose oxidase, we faced the following problem: The FAD molecules in this range also absorb

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light, something that covers up the TRIMID peak. In determining the TRIMID share of T-GOD, we used the fact that the FAD, after incubation at higher temperature, is separated from the enzyme. The FAD peak is superposed on the absorption bands of TRIMID; therefore, to detect TRIMID, we first of all separated the FAD in that the protein (500 µl each) was incubated for 2 hours at 50°C and that the solution was then chromatographed via a PD10 column. The enzyme fraction was then recognizable only after the protein peak at 280 nm.

The protein fraction was then illuminated in the flask twice for 10 minutes each and an absorption spectrum was recorded after each illumination. In Figure 3, one clearly recognizes a change in the absorption between 340 nm and 400 nm, in other words, within the range of the TRIMID bands.

The content of covalent bound TRIMID amounted to 8 ± 2 mole of TRIMID per mole.

The enzymatic activity of the modified protein was determined spectroscopically by means of the above-described enzymatic assays. For this purpose, we studied 50 µl of T-GOD that contained 8.73 µg of protein.

Using this method, we determined the enzymatic activity of the glucose oxidase solution employed. Figure 4 shows the kinetics of the enzymatic catalysis of the parent solution (GOD) as well as that of the modified enzyme (T-GOD).

A straight line was determined by means of linear regression via the linear portion of the curve. That straight line was used as a calibration straight line for the detection of the enzymatic activity of the particular glucose oxidase on the sensor.

The determination of the increase in absorption by means of linear regression for the case of T-GOD yielded a value of 0.989 min^{-1}. With the help of the Lambert-Beer law, one can determine

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from this the specific enzyme activity of the modified glucose oxidase of 34.92 units/mg. In comparison, the non-modified glucose oxidase has a value of 87.59 units/mg.

To observe the bonding of T-GOD on a surface and specifically on polyimide, the generated T-GOD was marked with [^{14}C]. For this purpose, glucose oxidase was first of all methylated reductively and was then immobilized on a polyimidized surface.

During reductive methylation, amino functions are mixed with formaldehyde and the resultant Schiff's bases are reduced. This highly specific reaction attacks only the amino groups of the lysine units in the protein as well as the N-terminus in the amino acid sequence. To avoid denaturing due to destruction of the disulfide bridges as well as the immediate reduction of the formaldehyde, one uses, as the mildest reducing agent, sodium cyanoborhydride, which leads to the N,N-dimethyl derivatives. This results in an almost complete mixing when one uses the six-fold excess of formaldehyde related to the protein mass.

Reductive methylation takes place in HEPES buffer (pH = 7.5); therefore, we rebuffered with 1 ml of T-GOD (4.22 mg/ml) via a PD10 column and we used the protein fraction for radioactive marking. We proceeded as follows:
800 µl of this protein fraction was placed in a light-protected reaction vessel (protection of the TRIMID group);

Addition of 6.5 µl of [^{14}C]-formaldehyde (= 200 nmol) with a radioactivity of 11.6 µCi;

Addition of 100 µl $NaCNBH_3$ (= 240 mmol/l);

Filling up with HEPES buffer to 1 ml;

Stirring for 3 hours at room temperature;

the product was chromatographed via a PD10 column in order to separate any excess formaldehyde.

The T-GOD fraction of the radioactively marked enzyme was examined for the protein content as described above. We got a protein concentration of 0.915 mg/ml. /12

To determine the degree of radioactive modification of T-GOD, we mixed in each case 5 µl of the individual fractions of [^{14}C]-T-GOD synthesis with 5 ml of scintillation solution (a mixture of 1080 ml toluene p.a., 5.4 g of 2,5-diphenyloxazol (PPO), 0.2 g of 2,2'-p-phenyl-bis-5-phenyloxazol (POPOP), 920 ml of Triton and 40 ml of glacial acetic acid) (Vortex) and we determined the radiation with a scintillation counter.

A radioactivity of 2,793 dpm (dpm = decompositions per minute) was found in the protein fraction. The correlation of these values with the results of protein determine according to Lowry of the radioactively marked protein yielded a figure of 597 dpm/µg.

The enzymatic activity of the radioactively marked protein fraction was again determined by means of the enzymatic assay.

For this purpose, we diluted 20 µl of the protein fraction with HEPES down to 200 µl, and of 50 µl of this solution (= 4.6 µg), we determined the enzymatic activity.

The evaluation showed an absorption increase of 0.27 min^{-1}, which corresponds to a specific enzyme activity of 18.19 units/mg. This means that the enzymatic activity is still present even after two modifications of the protein.

To observe the coupling of the protein upon a polyimidized surface, we polyimidized little cover plates made of glass, we then coated then with [^{14}C]-T-GOD and we measured the reactivity. The polyimidization of hydrophile surfaces, along with other methods, is an important basis for immobilization upon acoustoelectrical structural components.

Upon the little polyimidized glass plates, we applied 30-50 µl of the radioactively marked enzyme; after a defined action time, we illuminated and we then washed. Similarly treated but unilluminated little plates were also brought along as a control experiment.

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The washing procedure consisted of five times flushing the little plates with a solution of 50 mM PBS, 150 mM NaCl as well as 0.02% by volume of TWEEN 20. To determine the radioactivity, we put the little cover plates into the scintillation tubes, we covered then with 5 ml of scintillation solution, and after brief

mixing (Vortex), we determined the radiation of the polyimidized glass carriers. In Figure 5, we can see that the illumination after 30 minutes of action does not bring about any bonding upon the polyimide. The radiation of these little glass plates is roughly on the same order of magnitude as that of the unilluminated control samples. Only after considerably longer action times prior to illumination or partial drying by application of a vacuum -- which resulted in a completely dried protein film -- is there a covalent bonding of the protein upon the polyimide, something that can be recognized by the definitely higher radioactivity of the corresponding glass carrier.

A photoimmobilization of T-GOD upon polyimide is therefore possible, provided only little water is present in the protein matrix. In the absence of water, the degree of modification of T-GOD with TRIMID is too small so that all carbenes, generated by illumination, abreact with water and so that there is no measurable bonding upon the surface. These investigations furthermore show that the chosen washing procedure is suitable for removing nonspecifically adhering protein molecules from the polyimidized surface and to wash out the residual radioactivity.

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Claims:

1. Sensor to detect proteins according to the principle of the key-lock reaction consisting of a sensor body, one surface

of which is covered with a polymer layer where the receptor molecules of the key-lock reaction are bound to the polymer layer, characterized in that the bonding between the polymer and the receptor molecules is promoted by a photoreactive molecule that is bound in a covalent manner upon the receptor molecule and is inserted in a C-H bond of the polymer.

2. Sensor according to Claim 1, characterized in that the photoactive molecule is 3-trifluoromethyl-3-(m-isothiocyanophenyl)-diazirine (TRIMID).

3. Process for coating sensors according to one of Claims 1 or 2 with proteins where the surface of the sensor is modified and where a silane-containing promoter layer is applied upon the sensor surface and where a polymer layer is bound thereon, characterized by the following process steps:

   a) modification of proteins by bonding of carbene-generating molecules upon the lysine units of the amino acid sequences of the proteins;

   b) application of modified proteins upon the polymer layer;

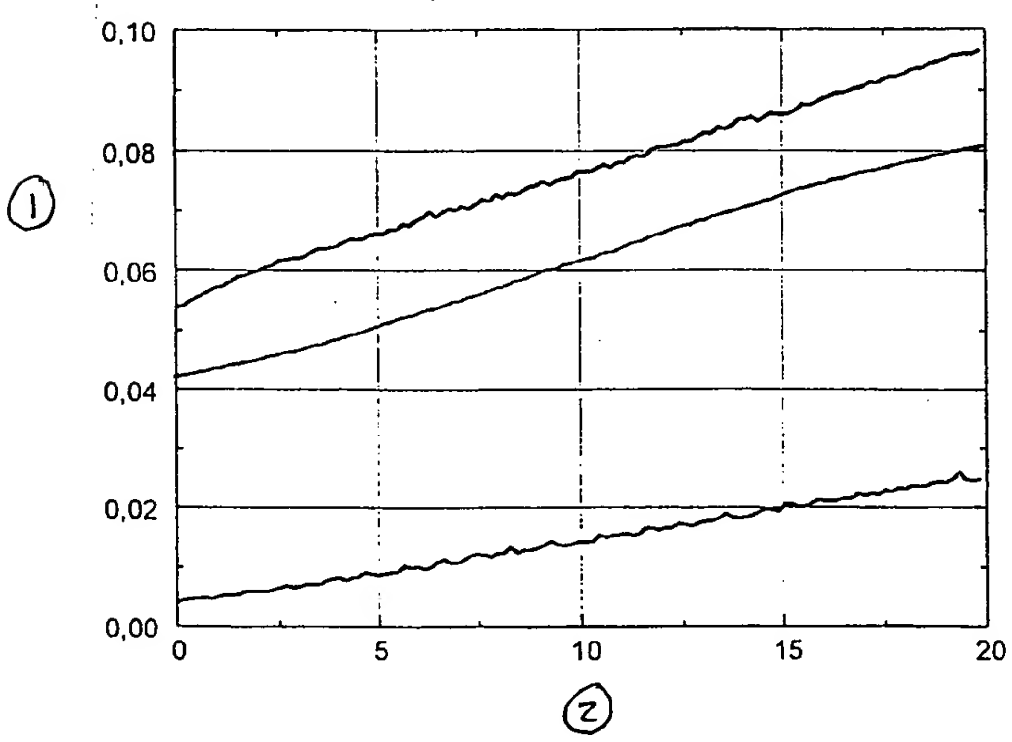
   c) partial drying of this layer and

   d) covalent bonding of the modified proteins upon the polymer layer by the action of ultraviolet light.

4. Process according to Claim 3, characterized in that the carbene-generating molecules are TRIMID.

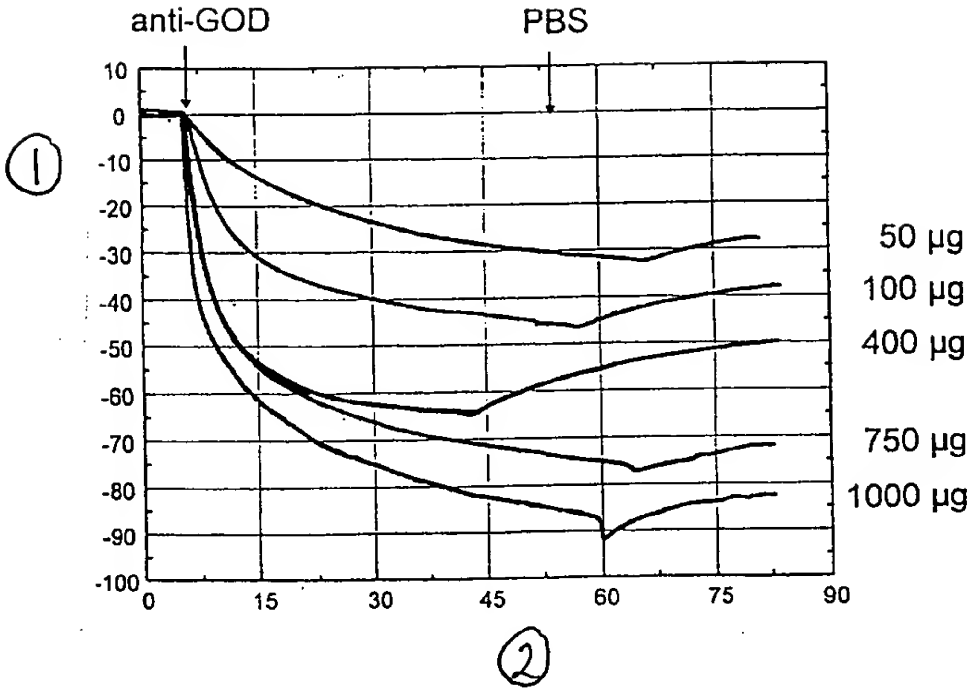
5. Process according to Claims 3 or 4, characterized in that the degree of drying of the layer is achieved by applying a vacuum.

Fig. 1 /15



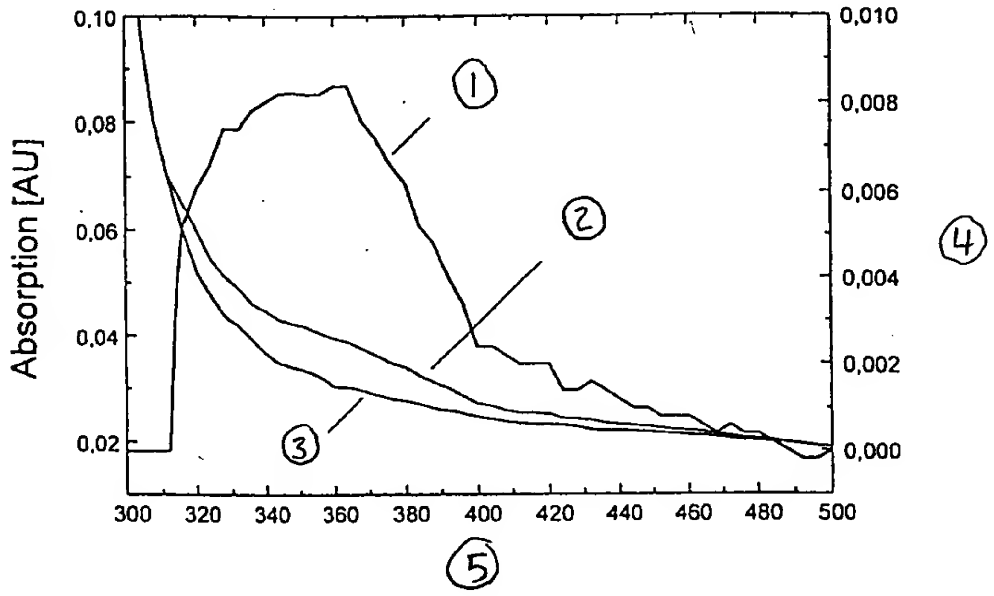
(1) Absorption increase at 520 nm [AU]; (2) Time [min]].

Fig. 2



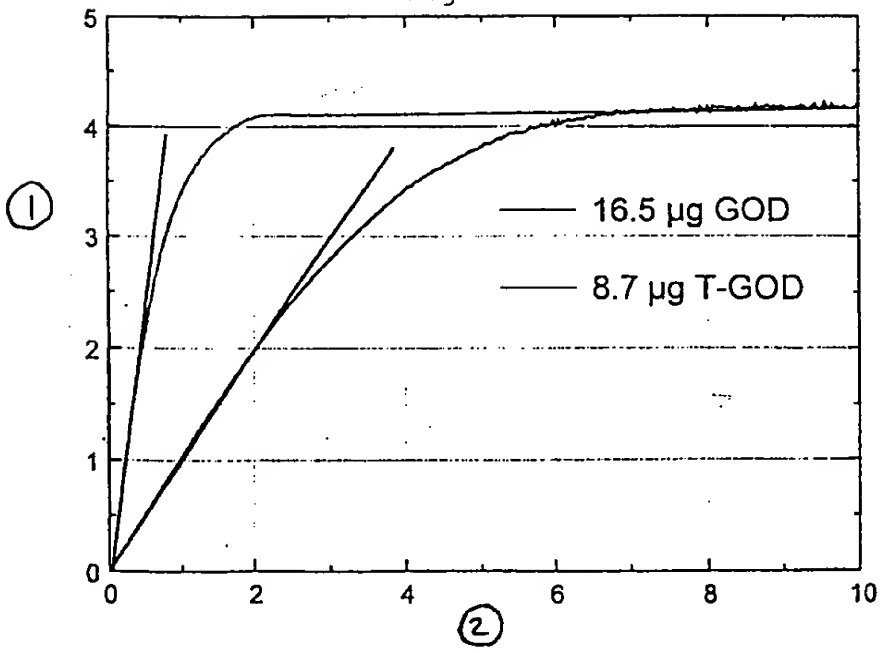
(1) Frequency change [kHz]; (2) Time [min]].

Fig. 3 /16



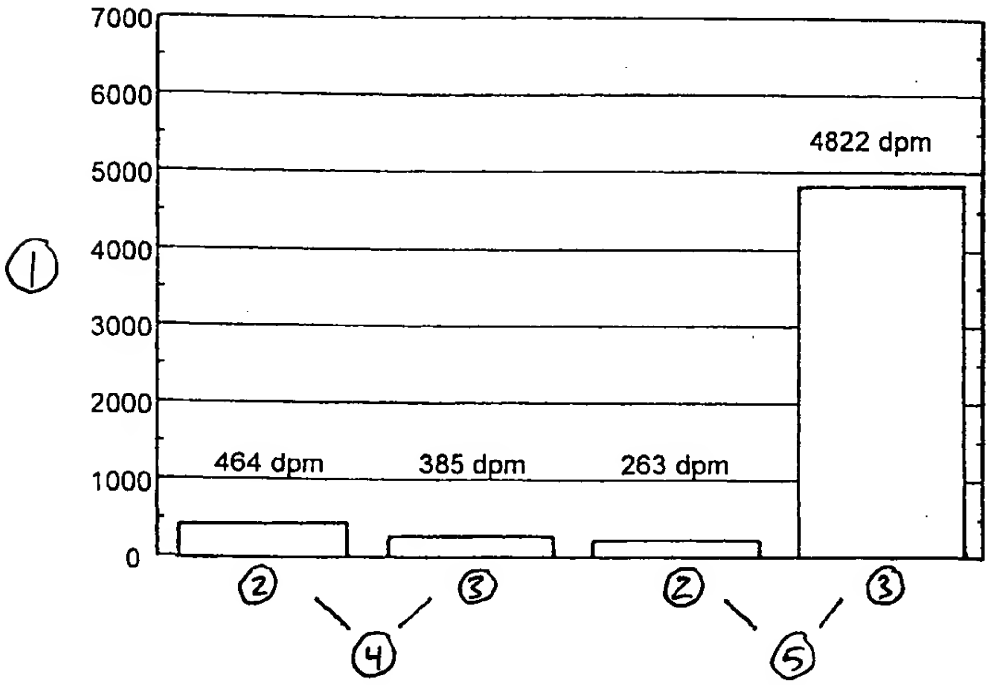
(1) Difference; (2) Prior to illumination; (3) After illumination; (4) Difference spectrum [AU]; (5) Wavelength [nm]].

Fig. 4



(1) Absorption increase at 520 nm [AU]; (2) Time [min]].

Fig. 5 /17



(1) Radioactive decays [dpm]; (2) Unilluminated; (3) Illuminated; (4) Action for 30 minutes; (5) Action overnight].